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Optimization of PCR parameters for microsatellite markers in barley

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Keywords

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PCR specificity
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ABSTRACT

Microsatellites are reliable and efficient DNA markers. They have been used for breeding and genetic diversity studies in barley. Although their use is robust compared to other markers, some PCR parameters should be optimized for their successful use. The aim of the present study was to discuss the importance of optimizing certain PCR parameters and to highlight some considerations for specificity and efficiency of PCR reactions. It was found that increasing $MgCl_2$ concentration from 1.5 to 2.5 mM and decreasing the reaction volume from 40 to 25 µl improved the specificity of the reactions. Increasing the Taq DNA polymerase amount from 1 to 2 units and employment of touchdown PCR procedure resulted in amplifications in otherwise unsuccessful reactions. It turned out that primer degradation was a common threat for PCR amplifications, and PCR primers should be checked on gels when an amplification problem is encountered. Some SSR reactions were found to produce artifacts such as stutter bands and heteroduplex DNA formations which could confound the genotyping with these markers. Use of different gel systems to score SSR data was also discussed. It was concluded that each SSR marker should be evaluated as a unique chemical reaction and results of amplifications should be evaluated carefully for a better use of SSR markers in barley research.

1. Introduction

DNA markers are effectively used in plant breeding and genetic identification studies. Because of their time-, labor- and cost-efficiency, PCR markers have been most common DNA markers of all. Simple Sequence Repeat (SSR = microsatellite) markers are a specific class of PCR markers with superior characteristics such as very high polymorphism rate and codominance (Hua et al., 2015). However, an efficient use of this marker system necessitates the solving of some specific problems unique to it as well as optimizing some general PCR parameters.

Microsatellite markers carry DNA repeats of 2-6 bases (Zalapa et al., 2012). Their high polymorphism rates are due to the number differences of these repeats; e.g., five repeats of AT bases vs. six repeats of AT. Therefore, length of different allele amplicons could be as short as two bases in some markers. Differentiating such similar length amplicons poses major challenges for most laboratories, which require use of specific gels or capillary systems for genotyping purposes.

Microsatellite markers are highly specific and robust. Although this marker system uses the sequence repeats, PCR primers used do not carry these repeats. Rather, primers are developed from low copy, unique sequences flanking the microsatellite sequences. Besides, SSR primers are generally long enough, i.e., 18-22 bases, to secure locus specificity (Waters and Shapter, 2014). Therefore, microsatellite markers mostly amplify single loci and are highly specific. Nevertheless, some locus specificity or amplification problems could be observed with these markers, which need to be remedied for an efficient use of this marker system in molecular biology. Stutter bands are a unique problem for microsatellite markers. Especially the markers carrying two-base repeats, e.g., AT or GC, have this problem. Stutter bands are generally shorter than the expected product and result from

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the "slippage" of Taq DNA polymerase on the repeat sequences (Hosseinzadeh-Colagar, 2016). Since these bands are generally one or two repeat units shorter than the actual product (Daniels et al., 1998), they may produce artifact bands which confound genotyping.

Barley genome maps have an abundance of SSR markers (Hearnden et al., 2007; Varshney et al., 2007; Ramsay et al., 2000; Li and Börner 2004). Some of them are of genomic DNA and some are of cDNA origins. This marker system has been used in barley for breeding and genetic diversity aims (Varshney et al., 2007). Especially the availability of draft genome sequence (Mayer et al., 2012) made SSR development an easy process for barley as in many other plants. The aim of the present study was to discuss some major problems encountered with SSR markers in barley and to show some solution strategies for them based on our experience in our DNA marker laboratory.

2. Material and Method

Genomic DNA was isolated using the Turkuaz DNA isolation kit (Keskin et al., 2014). Quality and quantity of DNA were evaluated using a 1% agarose gel and a spectrophotometer. The DNA concentration was adjusted to 50 ng/µL.

The primer sequences of the markers used were obtained from the GrainGenes database (https://wheat.pw.usda.gov/cgi-bin/GG3/browse.cgi?class=marker). As GBMS markers are patented, sequences of these markers for research use were provided by Marion S. Röder upon our request.

Standard PCR reaction volumes were 25 µl. In standard reactions, 250 nM each of the primer, 1.5 mM $MgCl_2$, 0.2 mM of each nucleotide, 1 unit Taq-DNA polymerase (Promega) and 50-100 ng genomic DNA were used. Changes to the conditions used for optimization are given in the descriptions of the gel pictures. Touchdown PCR was typically used in PCR reactions. After a hot start at 94 °C for three min., five touchdown cycles starting around the melting temperature of the primers and decreasing it 1 °C in each cycle were used since the annealing temperatures of 3-5 °C lower than the melting temperature of primers are recommended (Borah, 2011). Then, 32 cycles of 60 sec. at 94 °C, 60 sec. at 55-60 °C depending on primer, 60 sec. at 72 °C were performed followed by a primer extension at 72 °C for 5 min. Reactions were held at 4 °C after completion of PCR cycles. Loading dye was added and the reactions were run on gels. In regular procedure, the amplicons were resolved in 3% MetaPhor agarose (Lonza Catalog # 50180) with 1% TBE buffer. The amplified DNA was visualized by ethidium bromide added to the gels. Gel images were taken using Vilber Lourmat CN-08 DNA imaging system.

3. Results and Discussion

Although SSR markers are more robust and reliable compared to other PCR markers such as RAPD and AFLP, each SSR marker has its own PCR characteristics. Most SSR reactions could be carried out using some standard parameters, but it is not very uncommon to have amplification problems with certain markers. PCR parameters such as annealing temperature, $MgCl_2$ concentration, reaction volume, and amount of Taq DNA polymerase may need to be optimized before a successful use of SSR markers. A typical, good quality SSR marker in a barley F_2 segregating population is shown in Figure 1.

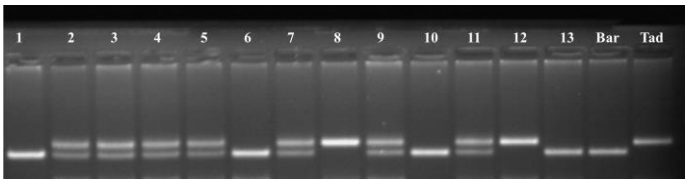

Figure 1. A typical SSR marker of Bmag0711 used to genotype a segregating F_2 progeny in barley. Lanes 1-13 are F_2 lines whereas lanes 14 and 15 are parents cvs. Baronesse and Tadmor, respectively.

3.1. Annealing temperature

Annealing temperature is one of the most critical parameters for SSRs as in other PCR markers. Annealing temperature should be 3-5 °C lower than the melting temperatures of primers (Borah, 2011). Melting temperatures are calculated and supplied by the primer synthesis software or primer manufacturers. However, the best annealing temperature should be determined experimentally. Lower annealing temperatures generally produce non-specific bands, which disappear at the appropriate annealing temperature. Too high annealing temperatures, on the other hand, may prevent annealing of the primer on the template, stopping the reaction completely (Figure 2).

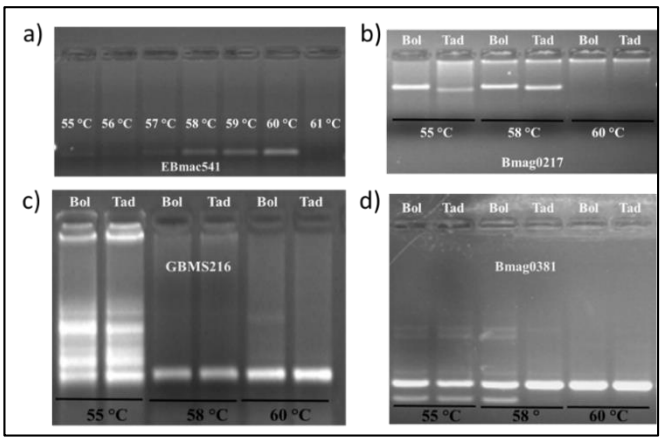

Figure 2. Effect of annealing temperature in four SSR marker. a) SSR marker EBmac541. Amplification increased up to 60 °C but stopped at 61 °C, b) Bmag0217. Reaction took place at 55 and 58 °C in Bol. (cv. Bolayır) and Tad. (cv. Tadmor) but stopped at 60 °C, c) GBMS216. Non-specific bands in Bol. and Tad. at 55 °C, which were eliminated at 58 and 60 °C, and intensity of the specific band increased at 60 °C, and d) Bmag381. Non-specific bands were produced at 55 and 58 °C in Bol. and Tad. which disappeared at 60 °C.

3.2. $MgCl_2$ concentration

Taq DNA polymerase needs $MgCl_2$ for its activity. However, Mg_2^+ ions interact with phosphate sugar backbone of nucleotides (Rahman et al., 2002) and consequently, $MgCl_2$ concentration can affect the specificity of the PCR reaction. A 1.5 mM concentration of $MgCl_2$ is typical in most PCR reactions in barley (Naeem and Mirza, 2018). However, higher concentrations of 2.25 mM $MgCl_2$ resulted in elimination of

non-specific bands in two of the three SSR markers shown in Figure 3.

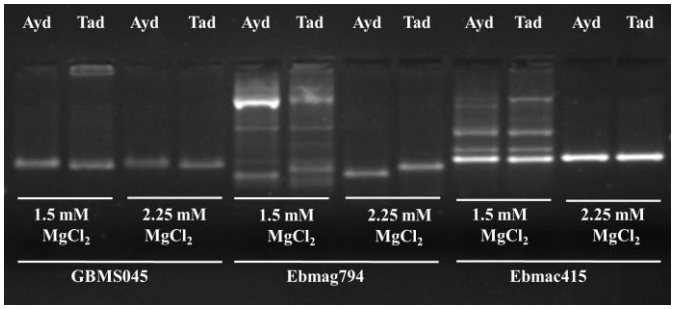

Figure 3. Effect of $MgCl_2$ concentrations on PCR amplification of Ayd. (cv. Aydanhanım) and Tad. (cv. Tadmor) reactions with SSR markers GBMS045, Ebmag794 and Ebmac415. In all reactions, especially in Ebmag794 and Ebmac415, amplifications of non-specific bands were lower in 2.25 mM $MgCl_2$ concentration compared to typical $MgCl_2$ concentration of 1.5 mM.

3.3. Reaction volume

PCR reactions of SSR markers are typically performed in 25 µl reaction volumes (Holton et al., 2002). But through the reaction dynamics, reaction volume could affect the amplification (Figures 4 and 5). Larger reaction volumes need extra amount of chemicals while lower volumes might require careful attention to certain procedures during the PCR process. In low volumes, mixing the reaction well and preventing the evaporation from the reaction volume which could occur during the opening of PCR tubes for the hot start practice of the reaction could be critical.

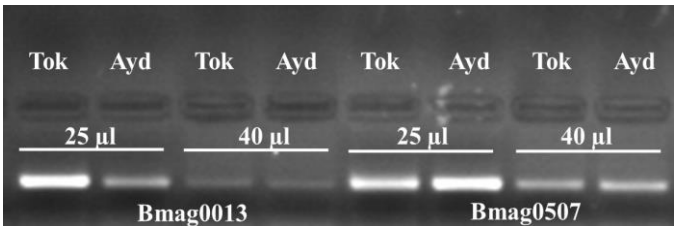

Figure 4. Effect of reaction volume on amplification of Bmag0013 and Bmag0507 markers in Tok. (cv. Tokak 157/37) and Ayd. (cv. Aydanhanım). The 25 µl reaction volume resulted in better amplification in both markers.

3.4. Amount of Taq DNA Polymerase enzyme

Taq DNA polymerase is the enzyme that carries out the DNA synthesis during the PCR. Generally, 0.5-1.0 units of enzyme is used in regular PCR reactions (Kandemir et al., 2010; Zhang et al., 2014). However, some extra amount of enzyme might promote amplification in certain SSR markers (Figure 5). Besides, because of low activity in some enzyme batches, e.g., as a result of long-term storage, use of some extra volume from the available enzyme batch could be a practical solution in some reactions.

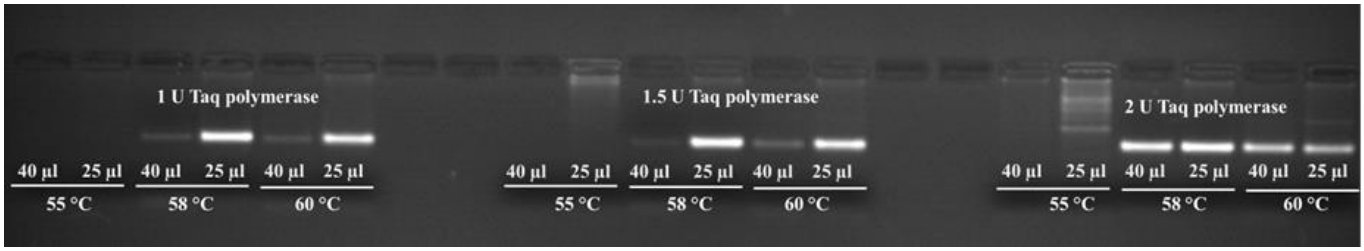

Figure 5. Effects of reaction volume, annealing temperature and amount of Taq DNA enzyme on amplification of barley GBM1030 marker in cv. Tokak 157/37. No amplification took place at 55 °C annealing temperature. Two units of enzyme resulted in better amplification at 25 and 40 µl reaction volumes at 58 and 60 °C annealing temperatures. When 1 or 1.5 units of enzyme was used, on the other hand, 25 µl reaction volume had better amplification.

3.5. Specificity of the PCR reaction and touchdown PCR procedure

First a few cycles of the PCR reaction are critical for the specificity of the PCR reactions, and the amplifications of the primer-template mismatches can be effectively amplified in the subsequent cycles (Korbie and Mattick, 2008). Therefore, a procedure called touchdown PCR in which the annealing temperatures a few degrees higher than the melting temperature of the primers are used, and the temperature is lowered by 0.5 or 1 °C in each cycle of this touchdown stage after which standard cycles of fixed annealing temperatures are used (Green and Sambrook, 2018). This practice also increases the amplification rate (Figure 6), and therefore is effectively used in many laboratories performing routine PCR reactions such as those in marker assisted breeding methods.

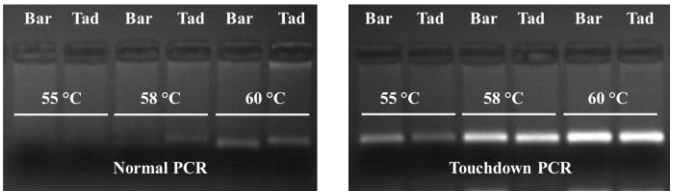

Figure 6. Effect of normal and touchdown PCR practices on amplification of GBM1275 SSR marker in Bar. (cv. Baronesse) and Tad. (cv. Tadmor) genotypes. In normal PCR procedure, only 60 °C annealing temperature produced clearly visible products while in touchdown PCR, 55, 58 and 60 °C temperatures all produced visible bands.

3.6. Amplification problems due to primer degradation

Especially in long term storage, degradation of primers, and consequent failure of PCR reactions, is not rare (Dawson et al.,

2010). When the primers that work well do not produce amplification, primer degradation should be suspected. Integrity of the primers should be checked in 3% MetaPhor agarose or 2.0-2.5% regular agarose gels (Figure 7). Elimination of primer degradation involves dissolving primers in TE buffer, rather than in water and keeping stock solutions at -20 or -80 °C, and decreasing the number of freezing-thawing cycles of primers (Jacquot et al., 2019).

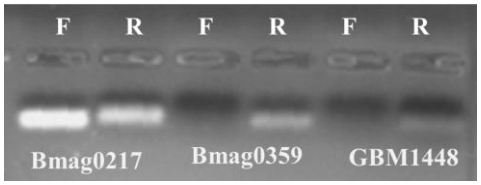

Figure 7. Checking the degradation of Bmag217, Bmag359 and GBM1448 primers on a 3% MetaPhor agarose gel. F: forward primer, R: reverse primer. For each primer, a 10 µl aliquot was loaded on the gel from 100 mM stock solutions. Both forward and reverse primers of Bmag217 were normal and they amplified DNA in PCR reaction while forward primers of Bmag359 and GBM1448 were degraded and they could not amplify.

3.7. PCR artifacts

3.7.1. Stutter bands

A major problem with SSR markers is the production of "stutter" bands. These bands are shorter than the real amplicon produced, and result in errors in genotyping (Guichoux et al., 2011). The cause of the stutter bands is the "slippage" by DNA polymerase during the amplification of the repeat sequence (Hosseinzadeh-Colagar, 2016). Stutter bands are common in dinucleotide repeats (Guichoux et al., 2011), and when possible dinucleotide repeats should be avoided during SSR development. Nevertheless, high polymorphism rates in dinucleotide repeats (Gadaleta et al., 2007) pose a dilemma involving difficulty of PCR on one hand and high polymorphism advantage of the marker on the other. Reducing the PCR cycles from 35 to 26 were reported to alleviate the stutter band problem in SSR (Bandelj et al., 2002).

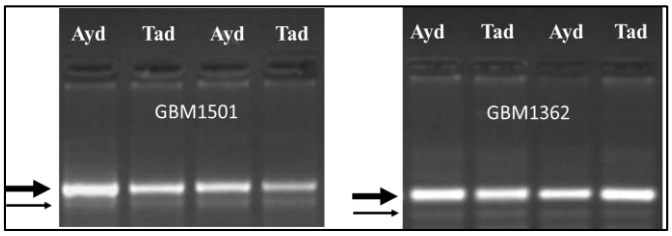

Figure 8. Stutter bands in PCR reactions of SSR markers GBM1501 and GBM1362 on a 3% MetaPhor agarose gel. Ayd: cv. Aydanhanım, Tad: cv. Tadmor. Large arrows show the true band of the markers while small arrows indicate the stutter bands.

3.7.2. Heteroduplex DNA formation

Heteroduplex formation is another confounding phenomenon for genotyping with PCR markers such as InDels (Insertion/Deletion) and SSRs markers. Combination of two different DNA strands of different alleles produce heteroduplex DNA (Hatcher et al., 1993). Formation of such a heteroduplex DNA in a PCR reaction is illustrated in Figure 9. Heteroduplex DNA bands do not become visible until about the 25th cycle of PCR reactions when one of the amplified fragments reach a critical level (Kulibaba and Liashenko, 2016).

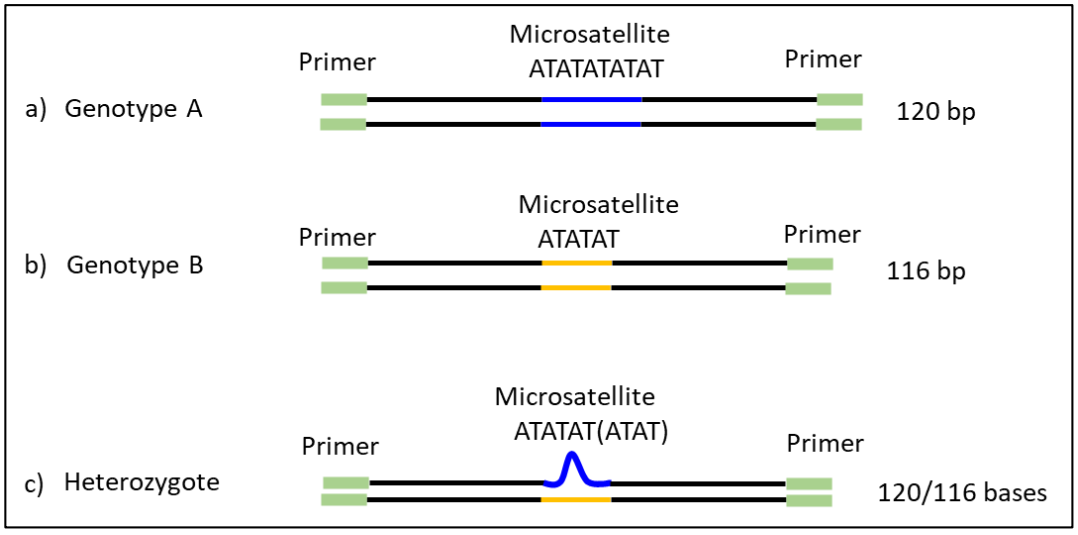

Figure 9. Heteroduplex formation in PCR reaction of heterozygous individuals. a) An SSR marker with AT dinucleotide repeat produces a 120 bp fragment in genotype A. b) The same marker produces a polymorphic allele of 116 bp in genotype B. The difference between the A and B genotypes is that the former has five tandem repeats of AT while the latter has only three. c) When DNAs from A and B alleles exist together in heterozygous individuals and when the two different-length alleles come together to form the double strand, a 120/116 base heteroduplex DNA is produced. Because of the conformational change, the heteroduplex has a mobility much smaller than the base numbers dictate, and the artifact band forms.

Heteroduplex bands appear in PCR reactions of some heterozygous individuals (Figure 10). These bands migrate slower than the heavier allele and result in difficulties with genotyping. Heteroduplex formation problem could be

alleviated by increasing Taq DNA polymerase amount and decreasing the number of PCR cycles (Michu et al., 2010).

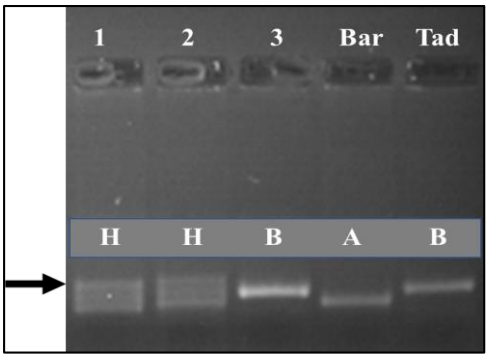

Figure 10. Extra PCR band produced in heterozygotes by SSR marker Scssr00103 is shown by the arrow. Bar: cv. Baronesse, Tad: cv. Tadmor. A: Baronesse allele, B: Tadmor allele, H: Heterozygote. 1, 2 and 3 are three F_2 lines from a Baronesse x Tadmor cross.

3.8. Resolution of SSR bands in gel systems

Most SSR markers have alleles differing in length by only a few bases, and their resolution in regular agarose gels may not be possible. Increasing the concentration of standard agarose gels up to 3% could improve the resolution of smaller DNAs such as those amplified by SSR reactions. However, preparation of a uniform gel is difficult at high concentrations. Special agarose gels such as MetaPhor Agarose, polyacrylamide gels or capillary system are required for genotyping of SSR fragments. Resolution of the same SSR marker products in barley using different gel systems is given in Figure 11.

Our lab has been using ethidium bromide added to 3% MetaPhor agarose gels, which allows multiple loading and checking of the same gel without staining and rinsing each time. Such a practice saves on time and cost spent for preparing these expensive gels. A gel image of seven sets of DNA loading is shown in Figure 12.

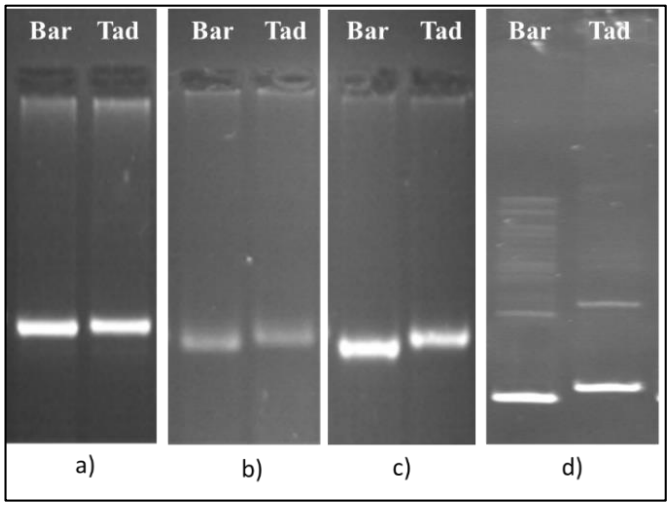

Figure 11. Resolution of Ebmag806 SSR marker bands amplified in barley using different gel systems. a) 3% agarose, b) 3% MetaPhor agarose, c) 5% MetaPhor agarose, and d) 20% polyacrylamide.

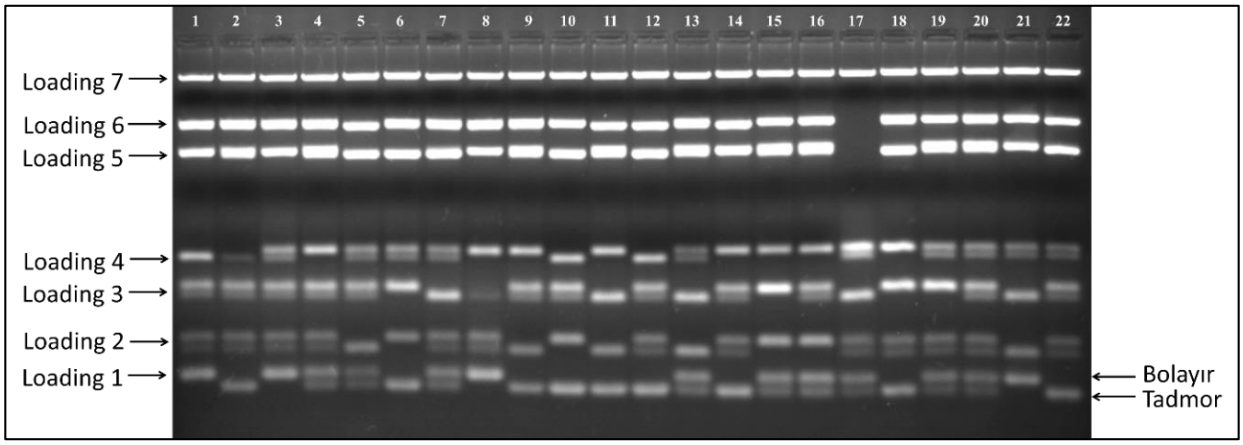

Figure 12. Evaluation of Bmag217 marker in a barley segregating F_2 population on a 3% MetaPhor agarose gel with ethidium bromide run in 1% TBE buffer. Seven sets of PCR products were loaded on this gel. Loading sets 1-4 could be scorable in this gel picture while sets 5-7 needed longer running. Cvs. Bolayır and Tadmor bands are shown by arrows on the lower right. This gel contains scoring of 154 plants including the parents.

4. Conclusion

Although SSR markers are highly reliable, care should be taken to optimize PCR reactions and to be aware of some considerations related to the PCR process and evaluation of the results. Annealing temperatures, $MgCl_2$ concentration, reaction volume and Taq DNA polymerase amount were the parameters affecting the specificity of the SSR reactions. Besides, using the touchdown PCR procedure and taking care of the common PCR artifacts such as stutter bands and heteroduplex DNA formation were also found to be critical in achieving an efficient and accurate results with SSR markers. Choosing a correct method for the resolution of PCR products seemed to be critical with SSR markers which have similar-size alleles. It could be concluded that each SSR reactions should be approached as a different chemical reaction with its own parameters that should be dealt with for a successful use of this potent marker system.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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